

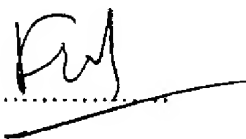
VERIFICATION OF TRANSLATION

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Declare that I am conversant with the French and English languages  
and that to the best of my knowledge and belief the following is  
a true translation of the International Patent Application No PCT/FR 98/01537.

Signature.....

A handwritten signature in black ink, appearing to be 'F. Lazard', written over a horizontal line.

Paris, January 25, 2000

## USE OF RECOMBINANT ENVELOPE PROTEINS FOR THE DIAGNOSIS OF THE DENGUE VIRUS

5 The present invention relates to agents for distinguishing the serological type of the virus responsible as well as the primary or secondary state of said infection, in the case of an infection by a virus of the flaviviridae.

10 The dengue viruses are transmitted to man by mosquitos of the genus Aedes. The World Health Organization estimates at one hundred millions the number of individuals affected annually by the dengue virus, of which several tens of thousands die, in particular in the intertropical regions where two billion persons at risk live. A growing number of tourists or employees of foreign companies are infected by travelling to countries where the disease is endemic.

15 Finally, the number of French people living in the DOM-TOM affected by this disease is counted in tens of thousands.

The disease, sometimes asymptomatic, is usually characterized by a high fever accompanied by headaches, nausea and articular and muscular pains which disappear after several days without leaving any consequences. The symptoms may in rare cases become more intense and lead to hemorrhagic symptoms (DHF), the end result of which may be fatal in the case of a hypovolemic shock (DSS). The criteria determining gravity are based on the hemoconcentration, thrombopenia, followed by cardiac insufficiency. At present an aggravation of the clinical picture is being witnessed with hepatic and neurological complications. The increase of the number of cases of DHF/DSS, in particular in adults, might have as common denominator the emergence of more virulent viral variants, doubtless due in part to hypertransmission and to a world-wide propagation of the viral species.

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The infectious agent is the dengue virus belonging to the Flaviviridae family, just like the yellow fever virus or the Japanese encephalitis virus (Monath andd Heinz, 1996). Four serotypes are known, dengues 1 to 4. These viruses have an envelope and possess an infectious single-stranded RNA of 11,000 nucleotides, associated with a capsid protein C. The viral envelope is composed of a membrane protein M and an envelope protein E. The viral

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RNA codes for a polyprotein of about 3400 amino acids which is resolved into three structural proteins and seven non-structural proteins NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 following co- and post-translational cleavages by viral and cellular proteases.

- 5 The proteins preM (M precursor), E and NS1 are produced in the endoplasmic reticulum and are glycosylated. The function of NS1 during viral replication is unknown.

The antibodies induced by the viral infection possess four biological properties which can be measured in vitro : (1) the type, complex or group specific antibodies directed against the envelope protein E ensure the neutralization of the virus; (2) the anti-NS1 antibodies participate in the cytolysis mediated by complement; (3) the antibody-dependent cell-mediated cytotoxicity or ADCC; and (4) the antibody-dependent facilitation. In vivo, it would seem that the specific anti-viral antibodies may prevent or stop an infection as a result of the neutralization of the virus and by lysis of the infected cells via complement activation. The hemorrhagic dengue might be a consequence of a poorly understood immunological phenomenon, no doubt involving a cascade of cytokines linked to the induction of cytotoxic cellular immunity of the CD4+ and CD8+ types which may result from a sequential infection by several serotypes. However, the appearance of hemorrhagic symptoms in primary-infected patients without opsonizing antibodies suggests that other factors such the gene pool of the individual or the particular virulence of the viruses also contribute to the serious symptoms of the disease (Monath and Heinz, 1996).

It was shown in the patent application No. WO 97/18311 that recombinant peptides derived from each serotype of the flaviviridae, in particular against the dengue virus, modified such that their carboxyl terminus substituted by a peptide containing 2 to 8 histidines, tryptophans or cysteines, and preferably 6 histidines, constitute, alone or in combination, an excellent immunogenic antigen which may serve as the basis for the constitution of a polyvalent vaccine. These recombinant peptides are preferably produced from insect cells, produced in the supernatant and readily purified on columns bearing a chelator owing to their histidine terminus. The content of the text of this application is incorporated in its entirety by reference in the present patent application.

The members of the flaviviruses (yellow fever, Japanese encephalitis, Zika, West Nile, dengue...) have common antigenic sites, revealed by the hemagglutination inhibition test (HAI) which forms the basis of the classification of these viruses. The  
 5 neutralization test, on the other hand, is more discriminating and can be used to distinguish between the viruses themselves and to classify them in subgroups of related viruses. On the basis of cross-reactions using polyclonal antibodies, the flaviviruses have been classified into 8 serocomplexes (Calisher et al., 1989) comprising 49  
 10 viruses. A score of viruses do not have sufficient specific antigenic relatedness to belong to one of these complexes. This classification has been confirmed since then by comparative analysis of the genes coding for the viral envelope protein or of the non-coding 3' end of several viruses (Marin et al., 1995, Pierre et al., 1994). Antigenic  
 15 subtypes have also been defined with the aid of monoclonal antibodies.

A major constituent at the virion surface, the E protein induces neutralizing antibodies and/or inhibitors of hemagglutination and induces protective immunity. The majority  
 20 of the neutralizing epitopes present on the E protein are discontinuous epitopes.

A low neutralizing activity was observed with the monoclonal antibodies directed against the preM protein. The capacity of the preM protein to induce protective immunity has  
 25 also been demonstrated in mice vaccinated with this protein.

The NS1 protein forms dimers in the infected cells, then is expressed at their surface, but it is also secreted into the extra-cellular medium in a hexameric form. It induces antibodies in  
 30 infected subjects. In the vaccinated monkey and mouse, this protein may even induce a protective immunity, the specific antibodies no doubt intervening in an ADCC phenomenon.

Anti-NS3 antibodies have been observed in infected subjects, although this protein has intracellular functions (protease, helicase, NTPase) associated with its localization near to the membranes of  
 35 the endoplasmic reticulum.

The NS5 protein is the viral replicase and few antibodies appear against this protein in infected subjects.

It is sometimes difficult to confirm the etiology of dengue fever when a patient presents an undifferentiated "dengue-like" type of febrile syndrome which may have as origin another arbovirus, viruses causing eruptive fevers, influenza, leptospirosis and even malaria. Only a laboratory examination can provide the diagnosis. The isolation of the virus by culture of mosquitos cells and its identification are lengthy procedures requiring the taking of an early blood sample which is stored at about 0°C. The RT-PCR method is not general and requires great technical ability.

The standard serological methods of HAI and complement fixation have been replaced by the more flexible and more sensitive ELISA procedure (Putnak and Henchal, 1990; Thongcharoen et al., 1993). More particularly, antibody capture assays of the MAC-ELISA type (for IgM antibody capture) or GAC-ELISA (for IgG antibody capture) have been developed for serological diagnoses of infection by the dengue virus (see for example Kuno et al., 1991). The IgA-type antibodies were not analyzed specifically in these assays.

The crude antigen used is a young mouse brain extract infected with the reference strains dengue 1 to dengue 4 or also insect (for example mosquitos) or mammalian cell lines infected with said viruses. In every case, a valid interpretation cannot be given on a single serum sample. It is absolutely necessary to take a second serum sample 10 to 15 days after the first. A diagnosis of dengue may only then be advanced if there is an increase in the titer of specific antibodies.

The method of IgM capture combined with the ELISA method makes it possible to detect the evidence of an active or recent infection and to confirm the diagnosis. The IgM are produced transiently during the primary infections as in secondary infections. However, the anti-dengue IgM titers are higher after primary reactions than after secondary reactions. The IgM/IgG ratio is thus used in numerous assays to differentiate a primary infection from a secondary infection. This method developed by Kuno et al. (1991), just like the ELISA method, is not specific for the serotype in question, particularly in the case of secondary reactions.

In a primary reaction, the antibody titer rises slowly to attain a modest level. The antibody titer is usually higher for the antigens

of the infecting virus than for those of the other flaviviruses. In secondary infections or in a subject previously vaccinated against yellow fever, the antibody titer rises rapidly to reach very high levels and cross-reactions are observed with a large range of flaviviral antigens. The antibody titer acquired at the primary infection is often higher than that of the antibodies induced during the secondary infection. Consequently, the serological result can, in any case, only establish a presumptive diagnosis.

At the time of a primary infection, the antibodies of the IgM type appear on day 5 after the start of the fever and persist for 3 to 6 months. The anti-dengue antibodies detected by the HAI assays appear about the 8th - 10th day and persist for several years. The neutralizing antibodies and ELISA are detectable as from the 6th - 8th day and persist for several decades (Deubel, 1990; Chan et al., 1975). At the time of a secondary infection, the IgM and the IgG appear more rapidly, on about the second day. The kinetics of IgA-type antibody formation mimic more or less those of the IgM.

Numerous attempts to fractionate viral antigens or to use peptides as antigen sources for the purpose of detecting type-specific antibodies have failed owing to lack of specificity or often as a result of lack of sensitivity, since the immune response is largely dependent on the host and the infecting strain. Furthermore, the existence of very many conformational epitopes at the surface of the envelope protein requires that antigens be used in the correct conformation.

No diagnostic method currently described or presently on the market, whether it be a "dot blot" assay for the detection of the IgM marketed by Venture Technologies Sdn (Malaysia) or by Integrated Diagnostics, Inc.(1756 Sulphur Spring Road, Baltimore MD 21227, USA) or the ELISA, IgM and IgG capture assays and immunochromatography developed and marketed by the PanBio Pty Ltd (116 Lutwyche road, Windsor K-4030, Australia) or even the immunochromatographic assay marketed by Chembio Diagnostic Systems Inc., 3661 Horseblock Road, Medford, NY 11763, USA) makes it possible to diagnose with sufficient reliability the serotype source of the infection as well as the primary or secondary nature of the infection (Cardosa 1988, 1991a, 1991b, 1992, 1993).

Since the antigens used hitherto have not been purified and individually identified, it was impossible to monitor the kinetics of the IgM and IgG or IgA antibodies directed against the protein supposed to be the most immunogenic at the time of infection or vaccination by a flaviviridae virus.

The diagnosis of dengue in a highly endemic area where several serotypes circulate must meet several requirements :

- a technical requirement based on rapidity, sensitivity and simplicity so that it can be performed on-site or at the bedside of the patient;

- a specificity requirement for typing the virus in a zone endemic for several arboviruses like dengue and the Japanese encephalitis virus in south-east Asia or like dengue and yellow fever virus in South America;

- a need to be able to diagnose in a febrile patient an arbovirosis by the presence of IgM;

- a possibility to diagnose the IgA whose kinetics of production and whose implication in the response to the infection and in the pathogenesis of the disease have not been characterized;

- a need to distinguish between primary and secondary infections in view of the possible association between a sequential infection and the risk of DHF.

In the patent application WO 97/18311 mentioned above, it was shown that polypeptides or glycopeptides of the different serotypes derived from protein E, preM, NS1, NS3 or NS5 of the flaviviridae, in particular of dengue, were able to constitute excellent immunogens, alone or in combination, capable of constituting a polyvalent vaccine against the dengue virus.

The present invention results from studies with the recombinant polypeptides of the invention WO 97/18311. This polypeptide can easily be purified in that it bears a deletion with the advantage of giving rise to a soluble protein, is secreted into the extracellular compartment and has a molecular structure similar to, if not identical with, that of the native protein. In addition, these polypeptides can easily be purified owing to the fact that they bear a histidine residue which has a particular specificity for metal chelate columns.

Another advantageous purification procedure for the viral antigens is affinity chromatography using as support an antibody specific for the protein to be purified, in particular a polyclonal or monoclonal antibody directed against one or more epitopes of the envelope protein. This procedure may be used alone or in combination with the previous one.

As the experiments described below show, the purification of these proteins in large quantity has made it possible to show that the recombinant polypeptides have a specificity of recognition both for the IgM and for the IgG, serotypes responsible for the infection without the risk of obtaining cross-reactions with other viruses.

The IgA also detected early after the infection show the same type of specificity.

The invention thus focusses on the use of these purified polypeptides or glycopeptides, derived from proteins E, preM, NS1, NS3 or NS5 of different flaviviridae, for the production of a diagnostic kit for the determination of the incriminating serotype in an infection by the dengue virus as well as on the primary or secondary nature of said infection. More particularly, the polypeptides or glycopeptides used in the invention bear at their carboxyl terminus 2 to 8 amino acid residues selected from histidine, tryptophan or cysteine.

Both for the flaviviridae in general and the dengue viruses in particular, the polypeptides are derived from the E protein of the four serotypes 1, 2, 3, 4 of said virus, 70 to 105 amino acids having been preferably deleted from their carboxyl terminus. When production is effected in diptera cell lines, this deletion makes it possible to produce the polypeptides by secretion, leading to easier extraction.

The 4 most widespread flaviviridae and from which it is possible to construct the polypeptides used in the production of a diagnostic kit are derived from the strains FGA/89, JAM/83, PaH/881 and 63/632 for the serotypes 1, 2, 3 and 4, respectively. A particular example of these polypeptides are those which contain a deletion of 100 amino acids and which are described in the application WO 97/18311.

The present invention also relates to a kit for the diagnosis of the primary or secondary state of an infection by one or several serotypes of the dengue virus and contains at least :

- 5       - one or more polypeptides, peptides or glycopeptides, labelled or not, derived from the proteins E, preM, NS1, NS3 or NS5 of different flaviviridae serotypes, in particular those of dengue, and purified for example if they bear at their carboxyl terminus 2 to 8 amino acid residues selected from histidine, tryptophan or cysteine;
- 10       - anti-IgG antibodies, labelled or not,
- anti-IgM antibodies, labelled or not,
- anti-IgA antibodies, labelled or not.

15       The polypeptide molecules or the antibodies can be used in a liquid medium. They are preferably bound to a solid support. If a "dot blot" assay is used, the polypeptides or the antibodies will be bound to supports of the nitrocellulose or polycarbonate type.

20       If an ELISA assay is used, the polypeptides or the antibodies will be bound to polypropylene or polycarbonate supports. If an agglutination assay is used, the polypeptides or the antibodies will be bound to latex-type supports.

25       If the polypeptides are bound to the support, the anti-IgG, anti-IgA or anti-IgM will be labelled or modified. If the anti-IgG, anti-IgA or anti-IgM are bound to a support, the polypeptides will be labelled or will be revealed by labelled or modified antibodies, specific for these polypeptides.

30       The specialist skilled in the art will know how to bind the polypeptide obtained and purified or the antibodies to the support of his choice as a function of the type of assay he wishes to use. The binding may be achieved by adsorption or may also be a covalent binding, for example, through the intermediary of carbodiimide. The different types of binding of the protein whether through a covalent bond or not to the supports for carrying out specific assays are performed according to the procedures known to the specialist skilled in the art (Johnstone and Thorpe, 1987).

35       A diagnostic kit according to the invention contains antibodies directed against IgG, IgA or IgM preferably of human origin in order to increase the specificity and sensitivity of the reaction with the antigen, optionally bound to a solid support. The

anti-IgG, the anti-IgA and the anti-IgM will be modified so that, after having reacted simultaneously with the antibodies bound to the polypeptides specific for each of the serotypes, the reaction products are detected and discriminated. In other words, the anti-IgG, anti-IgA or anti-IgM antibodies are modified in a different chemical manner such that the antigen/antibody modified human anti-antibody reactions are revealed respectively by a chemical molecule capable of emitting a signal, directly or indirectly by action on a substrate, which will be specific for the antigen IgG or IgA, on the one hand, or the antigen IgM, on the other.

By antigen is meant any one of the four peptides optionally comprising a deletion of 75 to 105 amino acids in the carboxyl terminal part and provided with from 2 to 8 amino acid residues as described above. The antigens may be homogeneous, i.e. relate to only one serotype, or heterogeneous, i.e. constituted of a mixture of at least 2 viral serotypes.

The polypeptides used, depending on the type of antigen or support may be used at concentrations included between 1 ng/ml and 1 µg/ml.

The specific signal making it possible to detect the existence of the antigen/antibody or antibody-anti-antibody reaction is a quite usual signal used in this type of reaction, namely a fluorescence, luminescence, colorimetric or radioactive signal.

A particular method of chemical modification of the antibodies or polypeptides according to the invention consists, for example, in chemically coupling a biotinyl group which then enables any substance linked to streptavidin to be coupled. The coupling may also be achieved through the intermediary of p-benzoquinone (FR No. 75377392 and the American patent No. 4, 925, 921).

The reaction specificity of the diagnostic kits containing the antigens described above makes it possible to consider the production of various kits which may be rapid assay kits to confirm a diagnosis or routine assay kits for performing epidemiological surveys.

Similarly, the constructions described above and illustrated by the example given below may be transposed to the expression and purification of the E envelope proteins or other polypeptides of the Japanese encephalitis and yellow fever viruses which, in turn, can

be used as antigens for the production of a diagnostic kit for infections by this same virus.

The examples given below, illustrated by the Figures, show the specific performances of the diagnostic kits of the present invention.

Figure 1 presents a Western blot of the envelope proteins of the 4 serotypes of the dengue virus. The mouse monoclonal antibody 4G2 described in Am. J. Trop. Med. Hygiene, 1982, No.3, pp. 548-558 (Gentry et al., 1982 directed against an epitope present on the envelope proteins of the four serotypes of the dengue virus was used for revelation. An alternative consists in using polyclonal antibodies prepared by hyperimmunization of mice immunized by the 4 serotypes of the dengue viruses.

Figure 2 shows the serological reactivity of serum of patients who had exhibited a febrile dengue epitope obtained by "dot blot" assay on nitrocellulose bands.

Figure 3 shows the serological reactivity of ascites of mice hyperimmunized against dengue 1 (HS1), dengue 2 (HS2), dengue 3 (HS3) and dengue 4 (HS4) or unimmunized mice (NS); against envelope polypeptides of dengue 1 (D1), dengue 2 (D2), dengue 3 (D3) and dengue 4 (D4) in a "dot blot"-type assay. C = negative control.

#### Example 1 - Development of a dot-blot assay for the serological diagnosis of dengue :

We have chosen to use as antigen the viral envelope protein, protein E, which is the preferred target of the protective humoral immune response in the infected host. We have made use of the baculovirus technology for the production of the E protein of the four serotypes of the dengue virus. The gene for the E protein was altered so that it was no longer retained in the intracellular membranes but secreted in a soluble form into the extracellular compartment, has a molecular structure similar to, if not identical with, that of the native protein, and is easier to purify as is shown in the protocol indicated in point 1.2 below. Furthermore, a sequence permitting adhesion to metal cations was placed at the end of the E protein gene, reducing the chromatographic

purification steps starting from cell supernatants (Staropoli et al., 1997).

### 1.1. Construction of the genes for the envelope protein.

A part of the sequence of the genes for the E protein of the four serotypes of the dengue virus was deleted which corresponds to the last 100 amino acids ( $\Delta E 100$ ) of the protein not detrimental to the maintenance of its three-dimensional structure necessary for the reactivity of the neutralizing antibodies. This deletion has the advantage of giving rise to a soluble protein which is secreted into the extracellular compartment, has a molecular structure similar to, if not identical with, that of the native protein and can be more easily purified.

The strains of dengue virus from which the E genes are derived are those shown in Table 2 of the application No. WO 97/18311.

The preparation of the E genes was achieved by RT/PCR from RNA extracts of mosquito cells *Aedes pseudoscutellaris* AP61 infected by the different viruses. A sequence coding for six histidine residues was placed at the end of the genes for the envelope protein. The recombinant baculoviruses AcD (1, 2, 3, or 4)  $\Delta E 100$  His 6 were obtained by homologous recombination in *Spodoptera frugiperda* Sf9 cell between the shuttle vectors pVLD (1, 2, 3 or 4)  $\Delta E 100$  His6 and the baculovirus AcRP23lacZ. The electrophoretic profile of the four recombinant E proteins corresponding to the dengue viruses indicates an apparent molecular mass of about 50 kDa for dengue 2,3 and 4 and of 52 kDa for dengue 1 (Fig. 1). These proteins are glycosylated and their molecular mass is reduced by about 4 kDa after treatment with endoglycosidases. It would seem that the two N-glycosylation sites of the E protein of dengue virus 1 are occupied whereas only one of the two potential sites is occupied for the viruses of the three other serotypes. The E proteins undergo a modification of their sugars in the secretory pathway which makes them resistant to the action of endoglycosidase H on the extracellular proteins.

### 1.2. Purification of the E protein by affinity chromatography on a metal ion column.

The presence of six histidine residues at the truncated C-terminus of the envelope protein confers on it the property of being

able to bind to divalent cations like  $\text{Ni}^{++}$  or  $\text{Co}^{++}$  and to be purified from cell supernatants in a single step. The histidine residue seems to be immunologically inert and may be maintained on the purified proteins for the immunizations. The binding step of the protein of interest to the cation support is crucial for the purification yield and requires the recovery of the cell supernatant at an appropriate time before cell lysis and a suitable treatment for concentrating the protein and for preserving its native state. Two methods of concentration have been tested : either precipitation of the protein with 40% ammonium sulfate or ultrafiltration. The two procedures enable the protein to be concentrated ten-fold and require a dialysis step to remove the competing ions of the extracellular medium and bring the solution to a pH and a salinity compatible with immobilized cation chromatography. After gentle shaking of the cationic resin in the protein solution, the resin is placed on a chromatographic column and washed with a salt solution. The protein is eluted from the resin under non-denaturing conditions with the aid of a salt solution containing 50 mM imidazole. The eluted protein usually shows a degree of purity higher than 95%. The protein is finally dialysed against PBS.

The quantities of protein produced, although varying with the serotype, are sufficient for the mass production of subunit proteins to be envisaged.

### 1.3. Serological test

#### a) with hyperimmunized mice

The E proteins for each serotype were calibrated by means of dot-blot (Cardosa et al., 1991b) by limiting dilutions against a monoclonal antibody of group 4G2. As negative control antigen we used the same purification procedure starting from a Sf9 cell supernatant infected by a wildtype baculovirus. The dilution selected for this "negative control antigen" is that corresponding to the mean of the dilutions determined for each serotype. The proteins diluted in PBS are deposited on a nitrocellulose membrane by aspiration with the aid of a manifold. The antigen concentration is determined by limiting dilutions against the 4G2 antibody and the dilution selected for each antigen is two-fold lower than that giving a positive response. The membrane is dried for 30 minutes at 37°C then preincubated in buffer to which is added 5% dehydrated

skimmed milk. Each nitrocellulose strip containing the four dengue antigens and the control antigen is incubated in a 1/40th dilution of ascites of mice hyperimmunized against dengue or patient serum in the incubation buffer. The antibodies are revealed by mouse anti-IgG or human anti-IgG, anti-IgA or anti-IgM labelled with biotin. The biotin is then captured by streptavidin coupled to alkaline phosphatase. The enzyme is finally revealed by a substrate.

Table 1 and Figure 3 show the results obtained when the nitrocellulose strips are incubated with the mouse ascites. In this experiment it seems that :

- the ascites of mice hyperimmunized against dengue 1 (HS1) recognizes only the corresponding antigen (D1) but the other ascites recognize all of the antigens to varying degrees;
- the ascites of mice not hyperimmunized (NS) does not recognize the dengue antigens (D1, D2, D3, D4) and the antigen prepared from wildtype baculovirus (c) is not recognized by the ascites of mice hyperimmunized against dengue.

Table 1 : Serological reactivity by "dot-blot" of mouse ascites against the recombinant envelope proteins of the four serotypes of the dengue virus.

| Mouse<br>ascites | Antigens |    |    |    |            |
|------------------|----------|----|----|----|------------|
|                  | D1       | D2 | D3 | D4 | Control(c) |
| Dengue 1 (HS1)   | 3+       | -  | -  | -  | -          |
| Dengue 2 (HS2)   | 3+       | 3+ | 1+ | 2+ | -          |
| Dengue 3 (HS3)   | 1+       | -  | 2+ | 1+ | -          |
| Dengue 4 (HS4)   | 1+       | 2+ | 1+ | 2+ | -          |
| Control (NS)     | -        | -  | -  | -  | -          |

b) with human sera

We tested 12 pairs of sera of patients suffering from dengue. The results are shown in Table 2 and in Figure 2. This test permits certain comments to be made :

- 5 - No background noise is observed on using purified proteins.
- The "dot-blot" assay shows more specific reactions of dengue virus 1 for suspected primary reactions of dengue 1 by the HAI method (titer < 320) whereas they are tetraivalent in patients having an undoubtedly secondary reaction of dengue 1 or dengue 3
- 10 (HAI>320).
- The sera were tested for the presence of IgM. The IgG competitors were absorbed previously on an "RF absorbing" product (Behring). The reaction proved positive and specific for the serotype in the case of patients suffering from primary dengue 1 but
- 15 weak or zero and unspecific for the viral serotype in patients suffering from secondary dengue.

The results of detection of the IgM are reported in Table 3. It seems that the diagnosis of the IgM is sufficient in the case of a primary infection to confirm recent infection and the viral

20 serotype. It will be possible to improve the sensitivity since the serum sample taken in the acute phase of the disease gives a negative or weakly positive result (Table 3).

Moreover, the sera of patients supposed to present a secondary dengue have low levels of IgM undetectable in the

25 present state of our procedure. The low level of IgM in the secondary reactions of dengue is a known phenomenon.

In another series of ELISA assays, the presence of IgA specific for dengue was observed, in parallel with the presence of IgM in the sera of patients suffering from dengue.

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Table 2. Serological reactivity of sera taken from patients who had shown a febrile episode due to dengue

| Sérum | D1   |     |    |      | D2   |      |    |      | D3   |      |      |    | D4   |      |      |    | Conclusion<br>(based on<br>DOT-ELISA) |
|-------|------|-----|----|------|------|------|----|------|------|------|------|----|------|------|------|----|---------------------------------------|
|       | IHA  |     | DB |      | IHA  |      | DB |      | IHA  |      | DB   |    | IHA  |      | DB   |    |                                       |
|       | SN   | DB  | SN | DB   | SN   | DB   | SN | DB   | SN   | DB   | SN   | DB | SN   | DB   |      |    |                                       |
| A1*   | <20  | <20 | -  | <20  | <20  | <20  | -  | <20  | <20  | <20  | <20  | -  | <20  | <20  | <20  | -  | DEN1 prim                             |
| A2    | 320  | <40 | 2+ | <40  | 40   | <40  | -  | 40   | 40   | 40   | <40  | -  | 80   | <40  | <40  | -  |                                       |
| B1    | <20  | <20 | -  | <20  | <20  | <20  | -  | <20  | <20  | <20  | <20  | -  | <20  | <20  | <20  | -  | DEN1 prim                             |
| B2    | 80   | 80  | 2+ | 40   | 40   | 40   | -  | 80   | <20  | <20  | <20  | -  | 80   | <20  | <20  | -  |                                       |
| C1    | <20  | <20 | -  | <20  | <20  | <20  | -  | <20  | <20  | <20  | <20  | -  | <20  | <20  | <20  | -  | DEN1 prim                             |
| C2    | 160  | 40  | 3+ | <20  | 40   | <20  | -  | 80   | <20  | <20  | <20  | -  | 80   | <20  | <20  | -  |                                       |
| D1    | <20  | <20 | -  | <20  | <20  | <20  | 1+ | <20  | <20  | <20  | <20  | -  | <20  | <20  | <20  | 1+ | DEN? sec                              |
| D2    | 1280 | 320 | 3+ | 1280 | <320 | <320 | 3+ | 1280 | <320 | <320 | <320 | 3+ | 1280 | <320 | <320 | 3+ |                                       |

|    |      |     |    |      |      |    |      |      |    |      |      |    |             |
|----|------|-----|----|------|------|----|------|------|----|------|------|----|-------------|
| E1 | <20  | NT  | -  | <20  | NT   | 1+ | <20  | NT   | -  | <20  | NT   | -  | DEN1 prim ? |
| E2 | 160  | 160 | 3+ | 80   | 80   | 2+ | 80   | 80   | 1+ | 160  | 80   | 1+ |             |
| F1 | <20  | <20 | -  | <20  | <20  | -  | <20  | <20  | -  | <20  | <20  | -  | DEN1 prim   |
| F2 | 320  | 80  | 3+ | 80   | <80  | 1+ | 160  | <80  | -  | 80   | <80  | -  |             |
| G1 | <20  | <20 | -  | <20  | <20  | -  | <20  | <20  | -  | <20  | <20  | -  | DEN1 prim ? |
| G2 | 80   | 40  | 3+ | 40   | 40   | 1+ | 80   | 40   | 1+ | 40   | <40  | -  |             |
| H1 | <20  | <20 | -  | <20  | <20  | -  | <20  | <20  | -  | <20  | <20  | -  | DEN ? sec   |
| H2 | 1280 | 320 | 3+ | 1280 | <320 | 3+ | 1280 | <320 | 3+ | 1280 | <320 | 3+ |             |
| I1 | 20   | <20 | -  | <20  | <20  | -  | <20  | <20  | -  | <20  | <20  | -  | DEN1 prim ? |
| I2 | 640  | NT  | 2+ | 320  | NT   | 1+ | 640  | NT   | -  | 320  | NT   | -  |             |
| J1 | <20  | <20 | -  | <20  | <20  | -  | <20  | <20  | -  | <20  | <20  | -  | DEN1 prim   |
| J2 | 160  | NT  | 3+ | 20   | NT   | -  | 160  | NT   | -  | 40   | NT   | -  |             |

|    |      |     |    |      |     |    |      |     |    |
|----|------|-----|----|------|-----|----|------|-----|----|
| K1 | <20  | <20 | -  | <20  | <20 | -  | <20  | <20 | -  |
| K2 | 1280 | 80  | 3+ | 1280 | 80  | 3+ | 1280 | 640 | 3+ |
| L1 | <20  | <20 | -  | <20  | <20 | -  | <20  | <20 | -  |
| L2 | 320  | 80  | 2+ | 320  | 80  | 2+ | 320  | 20  | 2+ |

DEN : antigen of the dengue virus

HAI : hemagglutination inhibition (reciprocal of the serum dilution); SN! : seroneutralization (reciprocal of the serum dilution); DB: dot-blot IgG (staining intensity at 1/40th dilution of serum)

• 1 signifies first serum taken in acute phase of the disease and 2 signifies second serum taken 10 - 15 days later in the convalescent period

? : presumption of a primary or secondary infection by the dengue virus

DEN ? : the infecting serotype responsible for the disease cannot be determined by the DOT-BLOT procedure (the sera K and L are derived from patients suffering from dengue 3 (virus isolation)).

Table 3 : Comparative study of the serological reactivity of the IgG and IgM in the serum of patients with dengue by means of the DOT-ELISA method

| Séra | DOT ELISA |         |         |         | IgG/IgM | IgG/IgM | IgG/IgM | MAC ELISA | CONCLUSION                                 |
|------|-----------|---------|---------|---------|---------|---------|---------|-----------|--|
|      | D1        | D2      | D3      | D4      |         |         |         |           |  |
|      | IgG/IgM   | IgG/IgM | IgG/IgM | IgG/IgM |         |         |         |           |  |
| A1   | -/-       | -/-     | -/-     | -/-     | +       |         |         |           | (based on the<br>IgM and IgG<br>DOT-ELISA) |
| A2   | 2+/2+     | -/-     | -/-     | -/-     | +       |         |         |           | D1 prim                                    |
| B1   | -/-       | -/-     | -/-     | -/-     | +       |         |         |           |  |
| B2   | 2+/2+     | -/-     | -/-     | -/-     | +       |         |         |           | D1 prim                                    |
| C1   | -/-       | -/-     | -/-     | -/-     | -       |         |         |           |  |
| C2   | 3+/3+     | -/-     | -/-     | -/-     | +       |         |         |           | D1 prim                                    |



|    |      |      |      |      |      |      |           |
|----|------|------|------|------|------|------|-----------|
| D1 | -/-  | 1+/- | 1+/- | 1+/- | 1+/- | 1+/- | D sec     |
| D2 | 3+/- | 3+/- | 3+/- | 3+/- | 3+/- | 3+/- | +         |
| E1 | -/-  | 1+/- | 1+/- | 1+/- | 1+/- | 1+/- | +         |
| E2 | 3+/- | 2+/- | 2+/- | 2+/- | 2+/- | 2+/- | +         |
| F1 | -/-  | -/-  | -/-  | -/-  | -/-  | -/-  | D1 prim   |
| F2 | 3+/- | 1+/- | 1+/- | 1+/- | 1+/- | 1+/- | ND        |
| G1 | -/-  | -/-  | -/-  | -/-  | -/-  | -/-  | D1 prim ? |
| G2 | 3+/- | 1+/- | 1+/- | 1+/- | 1+/- | 1+/- | ND        |
| H1 | -/-  | -/-  | -/-  | -/-  | -/-  | -/-  | D1 prim   |
| H2 | 3+/- | 3+/- | 3+/- | 3+/- | 3+/- | 3+/- | ND        |
| I1 | -/-  | -/-  | -/-  | -/-  | -/-  | -/-  | D sec     |
| I2 | 2+/- | 1+/- | 1+/- | 1+/- | 1+/- | 1+/- | +         |
|    |      |      |      |      |      |      | D1 prim   |
|    |      |      |      |      |      |      | +         |



## CONCLUSIONS

We have demonstrated that it is possible to develop a rapid  
 5 assay for dengue which might make it possible to distinguish, in the  
 case of an infection by the dengue virus, a primary infection from a  
 secondary infection in the same manner as the HAI or sero-  
 neutralization.

For the first time, and surprisingly in the light of the fact that  
 10 they are synthetic antigens, the diagnostic kits according to the  
 invention thus enable the serum of patients to be tested specifically  
 at the time of a primary infection .

Furthermore, this assay can be used to investigate the  
 specificity of IgM or IgA whose presence was revealed in the  
 15 standard MAC ELISA assay.

Other simple and rapid procedures used in some laboratories  
 for the diagnosis of the flaviviridae and in particular for the  
 dengue virus or other viruses (radial hemolysis, agglutination,  
 agglutination inhibition, immunochromatography, etc.....)  
 20 (Thongcharoen et al., 1993; Henchal and Putnak, 1990, Cardoso,  
 1991, 1992) are directly applicable to purified native and  
 recombinant proteins or to synthetic peptides.

The advantages demonstrated by the use of recombinant  
 polypeptides, native or natural proteins or synthetic peptides such  
 25 as, in this test, the envelope protein are :

- the study of the kinetics of appearance of the antibodies of  
 the IgG, IgA and IgM types,

- the low level or absence of cross-reactions between the  
 viruses of the same group by the use of native and purified  
 30 antigens,

- the absence of false positive reactions encountered when cell  
 extracts or crude cell supernatants are used.

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## CLAIMS

1. Use of one or more purified polypeptides or glycopeptides derived from the proteins E, preM, NS1, NS3 or NS5 of different flaviviridae serotypes, in particular of the dengue serotype, for the manufacture of a diagnostic kit for the determination of the serotype responsible for an infection and the primary or secondary nature of said infection.

2. Use according to Claim 1, characterized in that the purified polypeptide(s) or glycopeptide(s) bear at their carboxyl terminus 2 to 8 amino acid residues selected from histidine, tryptophan or cysteine.

3. Use according to Claim 2, characterized in that the polypeptides are derived from the E protein of the four serotypes of the dengue virus, from the carboxyl terminus of which 70 to 105 amino acids have been deleted.

4. Use according to Claim 3, characterized in that the polypeptides are derived from the E protein of the strains FGA/89, JAM/83, pah881 and 63632 for the serotypes 1, 2, 3 and 4, respectively.

5. Use according to the preceding Claims, characterized in that the polypeptides are soluble or bound to a solid support suitable for use in a serological assay, said assay being selected from the ELISA, DOT-BLOT, hemagglutination inhibition, radial hemolysis, agglutination and immunochromatographic assays.

6. Kit for diagnosing the primary or secondary nature of the infection by one or several serotypes of the dengue virus, characterized in that it contains at least :

- one or more polypeptides or glycopeptides derived from the proteins E, preM, NS1, NS3 or NS5 of different flaviviridae serotypes, in particular of the dengue serotype, and bearing at their carboxyl terminus 2 to 8 amino acid residues selected from histidine, tryptophan or cysteine;
- labelled or modified anti-IgG antibodies;
- labelled or modified anti-IgM antibodies;
- labelled or modified anti-IgA antibodies.

7. Kit according to Claim 6, characterized in that the polypeptides or the antibodies are soluble or bound to a solid support.

5 8. Kit according to one of the Claims 6 or 7, characterized in that the polypeptides are derived from the E protein of the four serotypes and from the carboxyl terminus of which 70 to 105 amino acids have been deleted.

10 9. Kit according to one of the Claims 6 to 8, characterized in that the polypeptide(s) or glycopeptide(s) are labelled and the anti-IgG, anti-IgM and anti-IgA are unlabelled.

10. Kit according to one of the Claims 6 to 9, characterized in that the IgG, the IgA and the IgM are human or animal immunoglobulins.

15 11. Kit according to one of the Claims 6 to 10, characterized in that the anti-IgG, anti-IgA and anti-IgM antibodies are modified such that simultaneous reactions of the IgG, IgA and IgM with the polypeptides, optionally bound to a solid support, are detected and discriminated.

20 12. Kit according to Claim 11, characterized in that the anti-IgG, anti-IgA and anti-IgM are modified chemically, and the reactions are revealed respectively by a chemical molecule capable of emitting a specific signal, directly or indirectly as the result of action on a substrate.

25 13. Kit according to Claim 12, characterized in that the signal is a fluorescent, luminescent, colorimetric or radioactive signal.

14. Purified antigen specific for the flaviviridae comprising a labelled polypeptide or glycopeptide derived from the proteins E, preM, NS1, NS3 or NS5 of a virus of the flaviviridae family.

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## ABSTRACT

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10 The invention relates to the use of one or more polypeptides  
or glycopeptides derived from the proteins E, preM, NS1, NS3 or  
NS5 of different flaviviridae serotypes, in particular of the dengue  
serotype, and bearing or not at their carboxyl terminus 2 to 8 amino  
15 acid residues selected from histidine, tryptophan or cysteine for the  
production of a rapid diagnosis kit for the determination of the  
serotype responsible for an infection by the dengue virus, and of the  
primary or secondary nature of said specific infection of the  
flaviviridae comprising a polypeptide or glycopeptide optionally  
20 labelled derived from the proteins E, preM, NS1, NS3 or NS5 of a  
virus of the flaviviridae family and anti-IgG, anti-IgM or anti-  
IgA, either unlabelled, or labelled or modified.

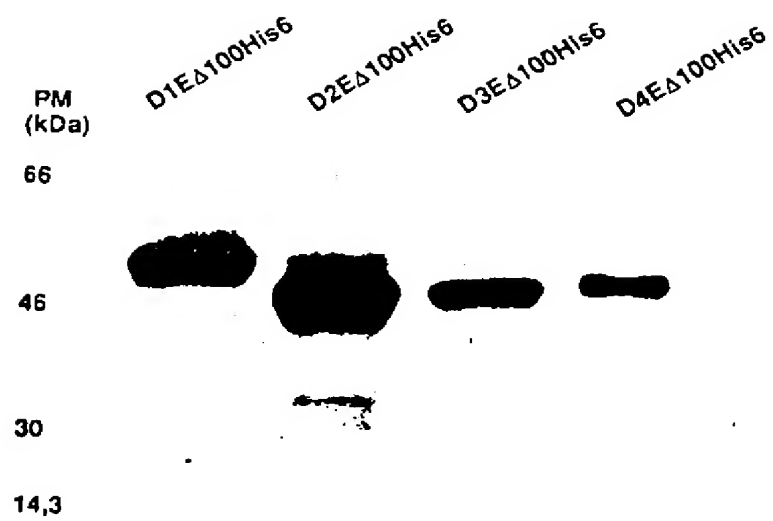


Fig. 1

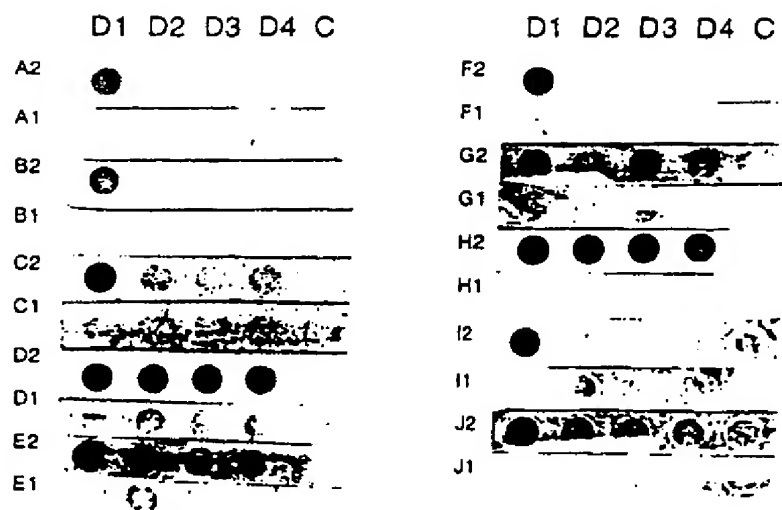


Fig. 2

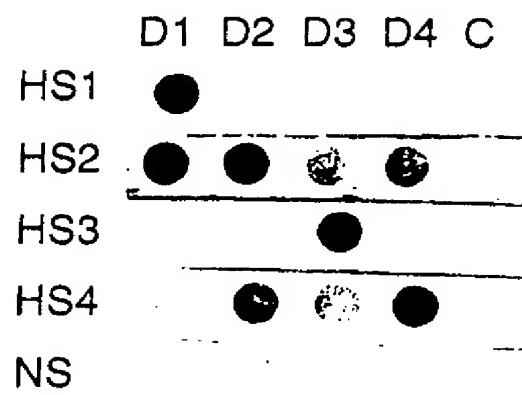


Fig. 3